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A semi-automated high-throughput approach to the generation of transposon insertion mutants in the nematode *Caenorhabditis elegans*

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ABSTRACT

The generation of a large collection of defined transposon insertion mutants is of general interest to the *Caenorhabditis elegans* research community and has been supported by the European Union. We describe here a semi-automated high-throughput method for mutant production and screening, using the heterologous transposon *Mos1*. The procedure allows routine culture of several thousand independent nematode strains in parallel for multiple generations before stereotyped molecular analyses. Using this method, we have already generated >17 500 individual strains carrying *Mos1* insertions. It could be easily adapted to forward and reverse genetic screens and may influence researchers faced with making a choice of model organism.

INTRODUCTION

In order to exploit the full genome sequence of *Caenorhabditis elegans*, a number of ambitious projects have been undertaken to address the function of all genes. Some, for example, have been based on the generation of ORFeome libraries (1,2), and include interactome (3) and structural genomics (4) projects. The *C.elegans* ORFeome library has also been used to carry out large-scale functional studies of gene inactivation by RNA interference (RNAi) (5), complementing previous screens based on other methodologies (6–9). Although such approaches give invaluable information about gene function, there is a clear need for the generation of stable mutant lines in as many *C.elegans* genes as possible. Following on from earlier work (10,11), two projects are currently underway in North America (12) (http://celeganskoconsortium.omrf.org/) and Japan (13) (http://shigen.lab.nig.ac.jp/c.elegans/index.jsp) to achieve this aim. As of September 2006, these projects had generated 2361 (G. Moulder, personal communication) and 2036 mutant strains, respectively. To these can be added the null alleles for several hundred genes produced using other PCR-based technologies (14,15) or generated in classical genetic screens and available from the central strain depository, the *Caenorhabditis* Genetics Center (http://biosci.umn.edu/CGC).

An alternative approach to the generation of mutants is via the use of transposons, a method that has been applied with great success in many model systems. Indeed in *C.elegans*, this was the first method that was used to isolate mutants in a systematic way (16). In this ground-breaking work, a frozen library of 5000 nematode lines mutagenized by the endogenous transposable element Tc1 was generated. Through a PCR-based screen, mutants of genes of interest could be recovered from the bank. The progeny of these mutant animals were then screened by PCR, to detect individuals in which there had been imprecise excision of Tc1 and thus inactivation of the gene of interest. This method had a number of drawbacks linked to the use of an endogenous transposon. More recently, a method using the mariner-like element *Mos1* from *Drosophila* has been established (17). In addition to being used as a mutagen in classical genetic screens, thereby accelerating greatly the speed at which mutations can be identified (18), it has also been used in a pilot-scale project to generate random insertions throughout the genome (19). Following on from the success of this pilot project that was carried out manually, as part of an ongoing collaborative effort to produce a large bank of mutants, we have developed a semi-automated high-throughput method for mutant production and screening that we describe here. With it, we have developed a capacity to handle several thousand nematode strains in parallel for multiple generations and have already generated >17 500 individual strains carrying *Mos1* insertions.

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MATERIALS AND METHODS

Strains and mutagenesis

The two strains of transgenic worms used, one carrying the substrate array (*oxEx229[Mos1:Pmyo-2:GFP]*) containing multiple copies of the *Mos1* transposon and associated with specific green fluorescent protein (GFP) expression in the pharynx, the other (*oxEx166[Phsp:Mos1Transposase;Punc-122:GFP;lin-15(+)]*) containing the coding sequence of the *Mos1* transposase under the control of a heat-shock promoter and a coelomocyte-specific GFP reporter have been described previously (17). These are crossed to obtain individuals carrying both arrays. Then double transgenic hermaphrodites, manually picked under a dissecting epifluorescence stereomicroscope (Leica MZFLIII), were subjected to heat-shock, resulting in expression of the *Mos1* transposon and consequently mobilization of *Mos1* as described previously (18).

Culture medium

The *Escherichia coli* strain OP50.1, available from the *Caenorhabditis* Genetics Center, was grown overnight at 37°C with shaking in Luria–Bertani (LB) supplemented with streptomycin. The culture was centrifuged for 20 min at 3220 *g*, the supernatant removed and the pellet resuspended in an equal volume of M9 supplemented with cholesterol (M9+) at a final concentration of 10 µg/ml to give the M9B medium that was also supplemented with streptomycin.

Liquid culture

Worms were individually sorted into 80 µl of M9B and cultivated at 20°C with agitation (900 r.p.m. on an orbital shaker) in a box humidified with damp paper towels. After 5 days, a 5 µl aliquot of each well in the 'A' plates (see Figure 1) was transferred manually with a multi-channel pipette to the 'B' plates containing 80 µl of M9B.

Worm sorting

Sorting was carried out using a Union Biometrica COPAS Biosort (Harvard Biosciences, Boston, MA), equipped with Reflex and Profiler modules, essentially following the manufacturer's instructions.

Well image analysis

Images of each well of a 96-well plate were acquired with a Flash Cytometer™ (Trophos, Marseille; see http://www.trophos.com/research/platform.htm). The images were subsequently processed using the ImageJ (NIH) image analysis software freely available from http://rsb.info.nih.gov/ij/. A binarized image was generated of each well and then the worms were positively discriminated from medium background, bacteria and dust (see legend to Figure 5). In the final step, particles were analyzed using the 'analyse particle' function. An area size filter was then applied in order to exclude particles with an area <0.015 mm^2 or >0.217 mm^2. The particles remaining in each well were assumed to be worms and their numbers in each well were automatically counted. The worm-counting results were used to automatically generate an Excel table. This table was filtered to include only those cells that contained at least three worms. This file was then read by a TECAN robot that selectively transferred the entire contents of the wells putatively containing worms to a new 96-well plate. The scripts used are available upon request.

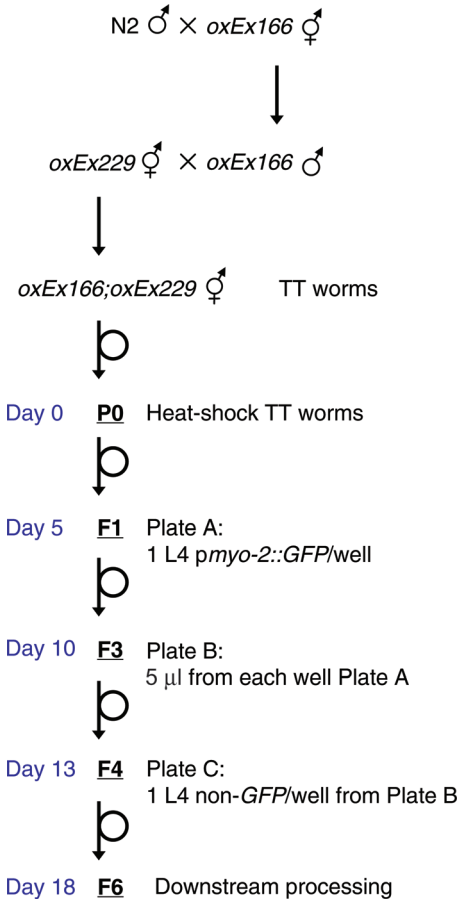

Figure 1. Simplified workflow for the upstream steps in the mutant generation protocol. Wild-type (N2) males were mated with hermaphrodite worms carrying the *oxEx166* transgenic array that contains the *Mos1* transposase and a coelomocyte-specific fluorescent marker. The resultant male cross-progeny were then mated with hermaphrodite worms carrying the *oxEx229* transgenic array that contains the *Mos1* transposon and a pharynx-specific fluorescent marker (18). The hermaphrodite cross-progeny carrying both the *Mos1* transposon substrate and transposase extrachromosomal arrays ('TT' worms), identified by the presence of both fluorescent markers (18), were picked manually and allowed to self-fertilize. The TT worms among their progeny were selected manually to give plates containing 150–300 young adult worms constituting the P0 generation. These were subjected to a heat-shock to induce transposase expression. Five days later, progeny of the first generation (F1) at the L4 stage carrying the *Mos1* substrate array were singled using the COPAS machine into 96-well plates, using size and fluorescence parameters for sorting. After a further 5 days of culture, worms of the F3 generation were transferred to fresh plates and 3 days later a single F4 non-GFP worm was transferred to a fresh plate using the Reflex system of the COPAS machine. These worms were allowed to reproduce to give the F6 generation that was then subject to downstream processing.

Worm lysis, PCR and gel electrophoresis

A 2.5 µl aliquot of each well from four 96-well plates was transferred to a single 384-well plate and 7.5 µl of fresh proteinase K solution (0.1 mg/ml in water) was added to each well. Plates were incubated at 65°C for 1 h before inactivating the proteinase K at 95°C for 15 min. Then to each

well 15 µl of a PCR mixture (9 µl H_2O, 0.25 µl of each of the oligonucleotides 5′-CAACCTTGACTGTCGAACCACCATAG-3′, 5′-TCTGCGAGTTGTTTTTGCGTTTGAG-3′ at 200 ng/µl, 0.25 µl of 25 mM dNTPs, 0.25 µl of Taq at 5 U/µl, 2.5 µl of 10× buffer and 2.5 µl of 50 mM $MgCl_2$) was added and the following PCR program performed: 3 min at 93°C, then 40 cycles of 30 s at 93°C, 33 s at 57°C, 40 s at 71°C, followed by 5 min at 71°C. Then 7.5 µl of loading buffer was added to each well and the entire contents loaded on to a 1.8% agarose gel.

Gel image analysis

After EtBr staining, an image of the 456-well gel corresponding to all the 384 wells of a PCR plate was acquired with a high-resolution CCD camera (Cohu Electronics, San Diego, CA). A predefined grid was applied to the gel image for helping the manual selection of positive lanes. Through the use of a JAVA program a click in each predefined region of the grid automatically loaded a '1' in the corresponding cell of a 16 × 24 Excel table. Conversely if a region of the grid was not selected, a '0' was automatically loaded in the Excel table. An Excel Macro then split the 16 × 24 table in four 8 × 12 tables each representing one of the four starting worm-containing 96-well plates used to load the 384 wells PCR plate. The resulting four Excel tables were then read by the TECAN robot which transferred the entire contents of each well flagged as positive to a well of a 24-well plate for subsequent culture. The scripts used are available upon request.

Transposition frequency

After each heat-shock, at the moment of the transfer of worms between plates A and B (Figure 1), a 5 µl aliquot from 28 randomly selected wells was spotted onto 3.5 cm plates containing standard NGM agar seeded with OP50. After 3–5 days at 20°C, from each plate, 10 non-GFP L4s (the F4 progeny from the original aliquot of F3 worms) were transferred to a fresh plate and cultured for one further generation. After confirming that none of the progeny was GFP-positive, 10 L4s of the F5 generation from each plate were transferred into PCR tubes containing 10 µl of proteinase K solution and subjected to lysis and PCR analysis as above. The 10 worms tested from each plate are all the progeny of a single F1 parent; by pooling 10 individuals at the F4 and F5 generations, the chance of not detecting Mos1 in the progeny of a Mos1-positive F1 worm is reduced. Since only F5 worms from F4 worms that gave no GFP progeny were used in the analysis, it is almost certain that none of the worms tested contains the Mos1 substrate array (*oxEx229*). The transposition frequency (%) is given by 100 × the number of positive wells/28.

RESULTS

Mos1-mediated mutagenesis

Protocols for the mobilization of the *Mos1* transposon in *C.elegans* are now well established. Transgenic animals carrying both enzyme and substrate arrays are generated by mating individuals carrying each array and these are then allowed to reproduce to give the parental (P0) generation (Figure 1). The next step involves inducing transposon mobilization by heat-shock of P0 worms carrying both enzyme and substrate arrays (18). After recovery, worms lay eggs that potentially contain the *Mos1* transposon integrated into their genome. It has been observed that animals that retain the substrate array at this F1 generation more frequently contain an *Mos1* insertion than those F1 individuals that have lost the array (18). As germline transposition is essentially limited to early meiotic nuclei of oocytes, all mutant F1 progeny obtained from parents in which transposition has occurred will be heterozygous for each independent *Mos1* insertion. With each subsequent generation, the proportion of worms heterozygous for a given locus will diminish as the strains are driven towards the homozygous (wild-type or mutant) genotype. After n generations following the F1, in the absence of any selection, in the population $(1/2)^n$ of the worms would be heterozygous and $[0.5 - (1/2)^{n+1}]$ homozygous mutants for a given insertion. To favor the recovery of mutants carrying homozygous insertions, we devised a scheme to isolate mutants after at least five generations (Figure 1).

Distribution and culture of mutagenized worms

To maximize the transposition frequency in a high-throughput setting, we used the Union Biometrica COPAS sorter to sort individual F1 worms carrying the substrate array. To generate a synchronized population, a sorting gate based on time-of-flight (correlated with size and hence age) was used to identify worms at the L4 stage. Since the substrate array is associated with specific GFP expression in the pharynx, the Profiler was used to identify those worms carrying it and discriminate them from those carrying the transposase array (associated with specific GFP expression in the coelomocytes) and from double transgenic worms carrying both (Figure 2). The sorting speed is dependent upon the density of the worms in the sample, and the proportion of individuals fitting the selection criteria, but under typical conditions, the machine filled a 96-well plate with worms in <3 min. This distribution was very accurate (Figure 3A).

In small-scale mutagenesis experiments, insertion frequencies as high as 75% can be obtained routinely (J. J. Ewbank and N. Pujol, unpublished data). In the current study, although a rate this high was occasionally achieved, it was more often <40% and sometimes even <10% (Table 1).

Following distribution, worms were cultured for 5 days at 20°C with shaking. In this time, worms that were properly fertile produced a large number of F3 progeny. In a typical experiment 81% ($n = 1824$) of wells contained a large population of worms. An aliquot of each well was then transferred to a well of a new 96-well plate with fresh medium and the worms cultured for a further 3 days. Again at this stage, good growth was generally seen and in a typical experiment 93% ($n = 1824$) of wells that contained a large population of worms at the F3 generation gave a large population of F4 and then F5 worms. Overall, therefore, on average 75% of wells at this stage contained worms, among which there were in theory 47% of individuals carrying two alleles of a given *Mos1* insertion, giving a combined 35% chance of recovering a single worm that was homozygous for a given insertion present in the F1 population.

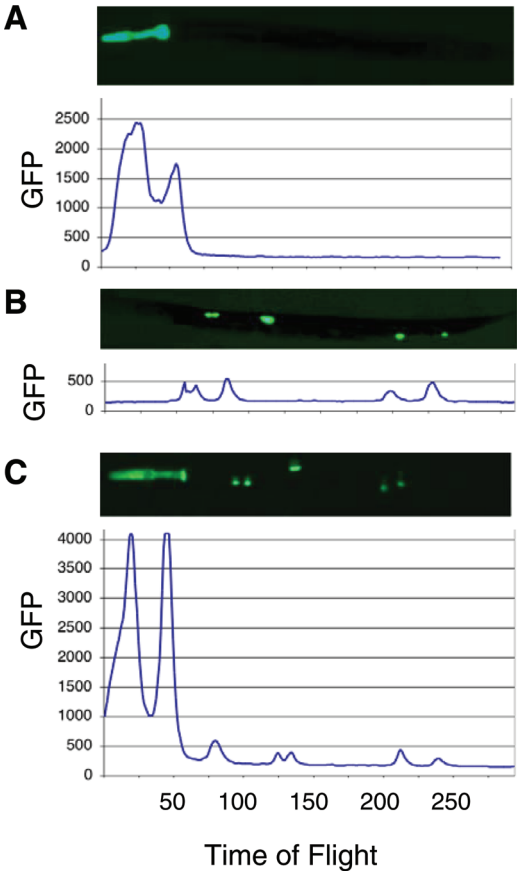

Figure 2. The COPAS Profiler can discriminate between worms carrying different transgenic arrays and can be used to sort worms of a desired genotype. Fluorescent micrographs (upper panels) and profiles (lower panels) for individual worms carrying the substrate array (**A**), the transposase array (**B**), associated with specific GFP expression in the pharynx and in the coelomocytes, respectively, or both (**C**). Fluorescence is measured in arbitrary but constant units. Time-of-flight is a measure of length (24).

Recovery of mutants

The COPAS sorter can be equipped with an accessory, the Reflex module, that allows recovery of worms from 96-well plates. Briefly, 200 µl of sheath fluid is pumped into a well and then all the contents are aspirated and passed through the analyzer. The standard program takes 55 min per 96-well plate. To improve this performance, a shorter custom Reflex cycle was instituted wherein only 100 µl of sheath fluid was added and a 60 µl sample aspirated from the well. This resulted in significant reduction in the time for each well to be treated and a whole plate could be analyzed in 36 min, increasing by >50% the number of plates that could be handled during an 8 h period (13 versus 8).

A stringent combination of standard and Profiler parameters was applied to select a single non-fluorescent worm from each well. These were automatically transferred to individual wells of a fresh 96-well plate. The COPAS machine was capable of performing this analysis and sorting in an accurate manner (Figure 3B). The vast majority of these worms was fertile and gave a large number of progeny (90% in a typical experiment; $n = 3840$) that were then processed to determine whether or not they contained a *Mos1* insertion.

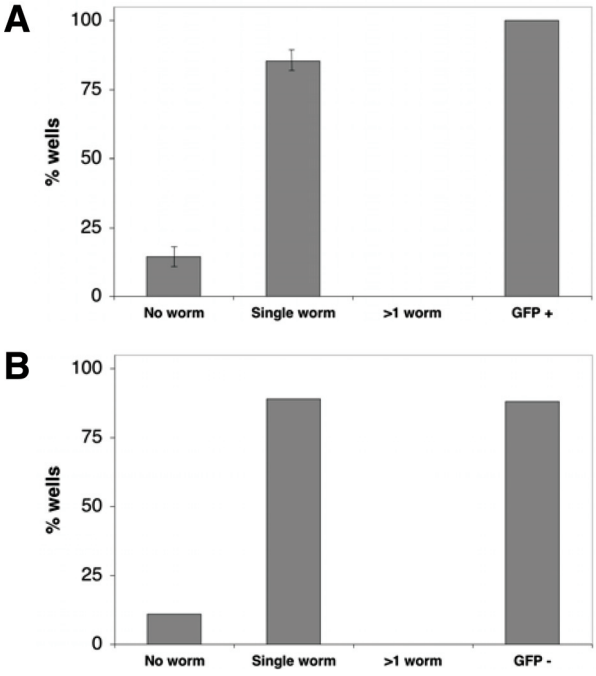

Figure 3. Sorting efficiency with the COPAS machine. (**A**) Sorting at the F1 stage. Bars represent the average for two independent trials of 96 wells, with error bars showing the standard deviation. The percentage of worms expressing GFP was calculated counting only those wells that contained a single worm; 100% of the singled worms had GFP expression in the pharynx. (**B**) Sorting at the F4 stage. Data are shown for a typical 96-well plate.

Table 1. Production figures for each week over a 10-week period

F1 worms	Transposition frequency[a] (%)	Number of *Mos1*-positive F5 strains (%)	Cumulative total[b]
3648	36.5	330 (9.0)	7676
3648	33.3	353 (9.7)	8029
3648	26.0	401 (11.0)	8430
3648	34.3	205 (5.6)	8635
3552	9.4	130 (3.7)	8765
3648	8.3	160 (4.4)	8925
3648	34.3	341 (9.3)	9266
3552	46.9	378 (10.6)	9644
3556	30.2	224 (6.3)	9868
4128	32.3	255 (6.2)	10 123

[a]The frequency was measured for a sample of 28 × 10 worms as described in Materials and Methods.
[b]Since the start of the project.

The overall workflow for this second part of the procedure is shown in Figure 4. During the initial stages of the project, before final protocol optimization, when the proportion of empty wells at the F6 generation was relatively elevated, the first step was to capture an image of each 96-well plate using a Flash Cytometer™. This system allows snapshot acquisition of entire wells (0.2 s/well) followed by digital image analysis. For the current project, publicly available image analysis software was used to allow automated recognition of nematodes (Figure 5). In this way, the wells that contained at least three worms were identified. The information regarding full and empty wells was then transformed into

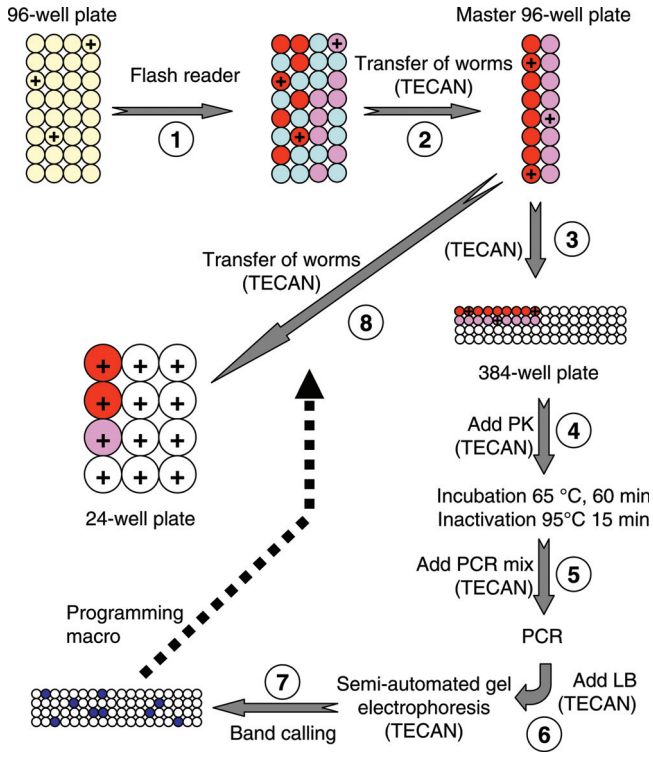

Figure 4. Downstream processing workflow for F6 generation worms. The numbered solid arrows indicate the successive steps. In this idealized schema, in the first step (1) individual 96-well plates containing worms in liquid culture were analysed with the Flash cytometer™ to identify wells full of worms (red and pink) and those that were empty or that contained only a few worms (light blue). The contents of each well determined to contain worms was transferred to a fresh 96-well plate (2). A small aliquot of each well from four such master plates was then transferred to a 384-well plate (3). A proteinase K (PK) solution was added to each well (4) and the plates incubated at 65°C for 1 h to lyse the worms before heat-inactivating the proteinase. A PCR mixture containing *Mos1*-specific primers was added (5) and a standard PCR performed. Electrophoresis loading buffer (LB) was then added to each well (6) and the contents of each well were then transferred robotically to a 456-lane agarose gel. After migration, an image of the gel was taken and the lanes containing *Mos1*-specific PCR amplicons identified (7). This information was related back to well addresses on the 384-well plate, allowing the *Mos1*-positive samples (indicated in dark blue) to be identified. This information was then used to cherry-pick the remaining contents of the master 96-well plates onto 24-well agar plates spotted with bacterial food (8). After 3–5 days, to allow the recovery and reproduction of the worms, these plates were then shipped to remote laboratories for analysis. Wells marked with a cross represent *Mos1*-positive samples. Only parts of 384-, 96- and 24-well plates are shown for clarity. Steps (1) and (2) were eliminated when the proportion of worm-containing wells exceeded 70%.

a programming macro to command a Tecan™ robot. The robot cherry-picked an aliquot of liquid from wells determined to contain worms, transferring it to a master 96-well plate. As the efficiency of the early steps improved, this part of the process became unnecessary and the plates containing worms of the F6 generation were processed without well redistribution.

Assaying for the presence of *Mos1*

A small aliquot of each well was taken from the 96-well plates and transferred to a 384-well plate. The worms in each well of the 384-well plate were then lysed and a PCR to amplify specifically the *Mos1* sequence was carried out directly in these plates (see Materials and Methods for details). The resulting mixture from each well was then loaded robotically onto a single 456-lane agarose gel. After electrophoretic migration and ethidium bromide staining, an image of the gel was captured. At the early part of the project, band-calling, to flag positive lanes contain the *Mos1*-associated PCR product, was done entirely manually. Subsequently, a point-and-click interface was developed to accelerate this process and make it less error-prone (Figure 6). In both cases, either manually or semi-automatically, the information regarding *Mos1*-positive wells was used to construct a new macro to allow cherry-picking of worms from the 96-well plates onto 24-well agarose, suitable for shipping. The subsequent characterization of the individual *Mos1* insertion sites was carried out by remote laboratories and will be the subject of a future publication.

The production figures for a 10-week period during which the cumulative total of *Mos1*-positive strains passed the 10 000 mark are presented in Table 1. Surprisingly, there was no apparent correlation between the rate of transposition measured in the F3 generation and the final yield of *Mos1*-positive strains (Figure 7; $y = 0.25\times$, $R^2 = 0.3$ for a linear curve fit). Overall, the procedure was relatively inefficient, with an average of 7 *Mos1*-positive strains obtained for each 96-well plate of individual F1 worms. This low yield was counterbalanced by the capacity to process very large numbers of individual clones in parallel; on average more than 3650 strains (incoming or outgoing, 7300 total) were handled per week during this period (Table 1). So, despite the low yield, more than 17 500 strains have thereby been generated to date.

DISCUSSION

At present, combining the results from systematic programmes and traditional genetic screens, there are mutants for roughly a quarter of *C.elegans* genes. The project we describe here is intended as a complement to these, as the *Mos1* insertion mutants can serve as the starting point for the engineering of specific genomic loci, allowing, for example, the production of specific mutant alleles, reporter strains or TAP-tagged proteins. The possibility of using transposon insertion mutants to introduce predetermined point mutations into the *C.elegans* genome was first demonstrated nearly 15 years ago (20). More recently, methods for homologous gene targeting (21) and a general strategy for gene conversion (22) have been described.

The feasibility of generating a large bank of *Mos1* insertion mutants has been established previously (19). That study showed that, apart from the rDNA locus on chromosome I that constitutes a hotspot, there was no particular insertion bias for *Mos1* in the *C.elegans* genome. But unfortunately, although 914 independent random *Mos1* insertion mutants were generated, roughly one-fourth of the identified insertions could not be found when frozen strains were thawed. This was suggested to be linked to the precocious freezing of heterozygous strains (19). Given this disappointingly low rate of recovery, we adopted a more laborious strategy that

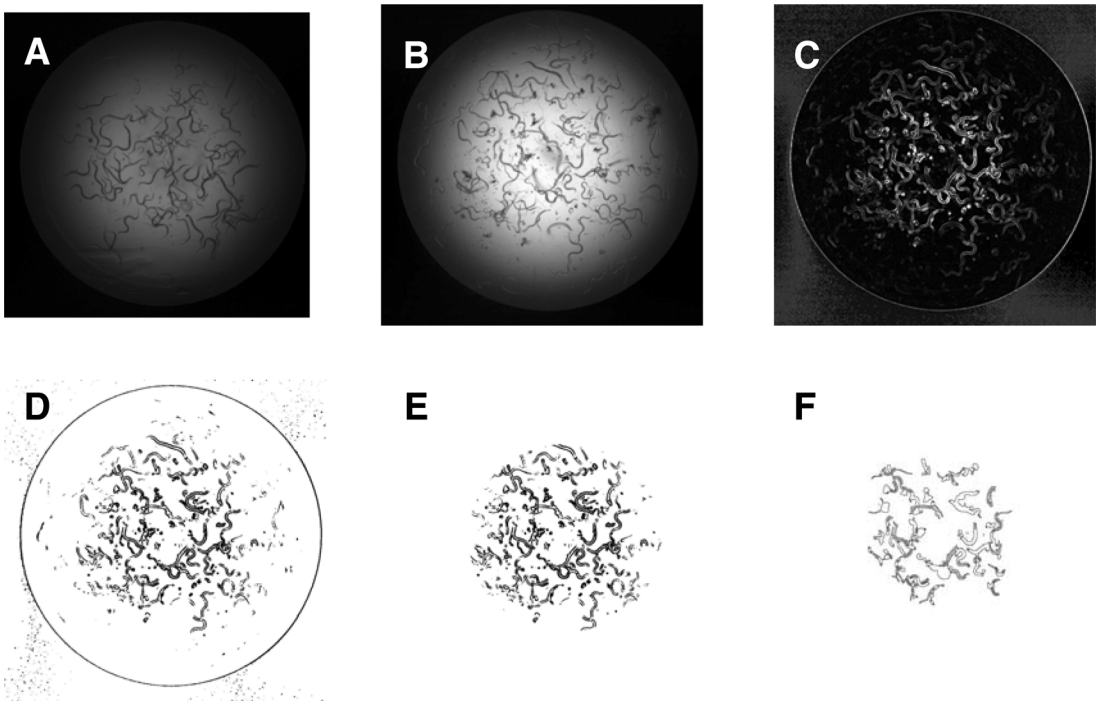

Figure 5. Successive steps in image analysis to determine whether a well contains worms. An image of each entire well of a 96-well plate was acquired automatically (**A**) and processed in successive steps using the ImageJ (NIH) software: overall light and contrast enhancement (**B**), loading of a circular region and inversion of gray level at each pixel inside the region (**C**), binarization on the basis of gray level at each pixel in the entire image (**D**), removal of all the selected particles outside the loaded region (**E**) and removal of all the particles shorter than a minimum size (**F**).

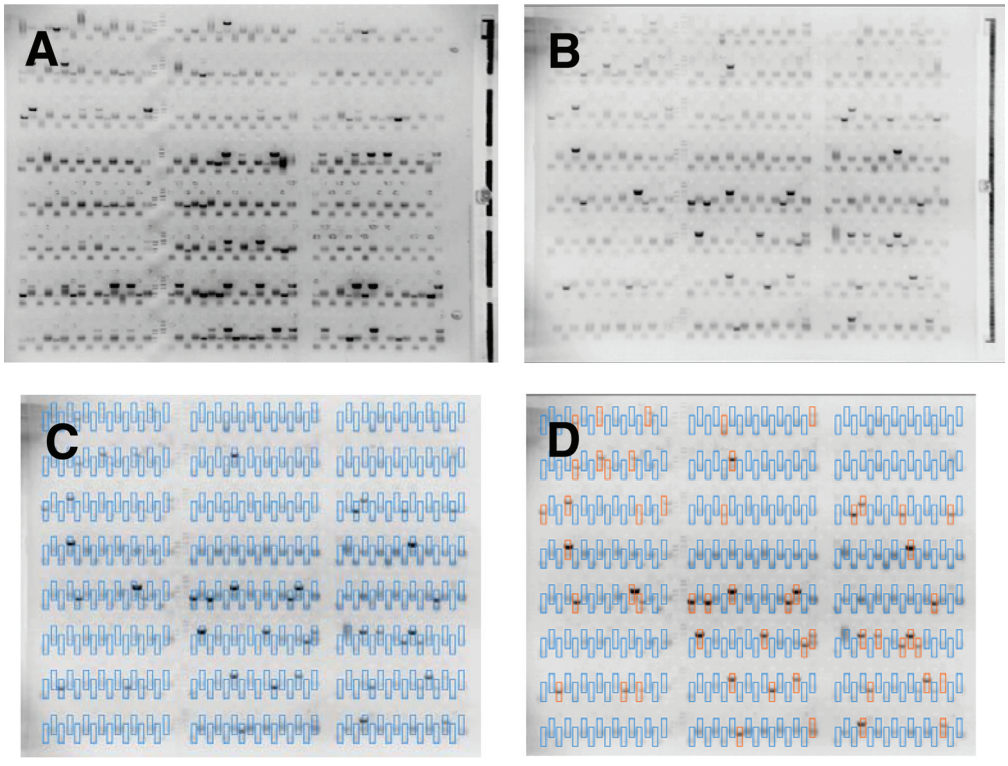

Figure 6. Identification of *Mos1*-positive samples. Samples were loaded on an agarose gel and subject to electrophoretic separation. After EtBr staining, an image of the gel was captured. Results for two representative gels are shown (**A** and **B**). A predefined grid was overlaid on the image, in this case the gel shown in B (**C**) and lanes containing a *Mos1*-specific PCR amplicon identified, as shown by the red boxes (**D**).

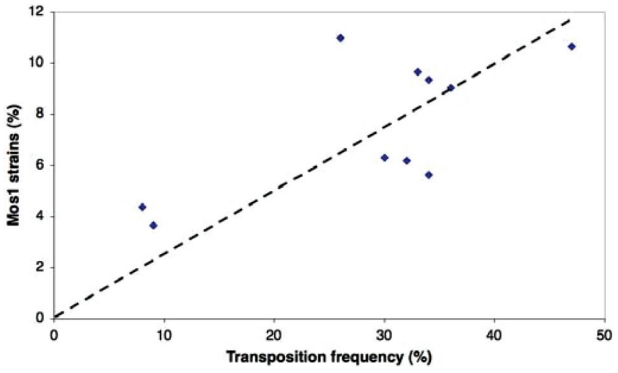

Figure 7. Relationship between the frequency of transposition and of *Mos1*-positive strains obtained. The percentage of transposition measured in the F3 generation for a sample of worms from each heat-shock is plotted against the percentage of wells giving a *Mos1*-specific PCR product at the F6 generation. The figures are taken from Table 1. The dotted line is a linear curve fit. The coefficient of determination for the curve fit is 0.3, indicating a lack of significant correlation.

drives the strains to homozygosity before they are cryopreserved, thereby circumventing the problem. A corollary of this is that the number of strains handled had to be increased 3-fold to counterbalance the fact that only 35% of single worms selected at the F4 stage would be homozygous for a given insertion present in the F1 population. Indeed, almost half (46.9%) of the singled F4 worms will have completely lost a given insertion. Additionally, there is the possibility of certain insertions being counter-selected. For example, if an insertion provokes a lethal recessive mutation, such that homozygous mutants do not reproduce, then at the F4 stage, 71.4% of individuals would be wild-type and only 28.6% heterozygous for the lethal mutation. Taken together with the relatively low transposition frequencies obtained, this meant that large numbers of samples had to be manipulated (Table 1). By combining the use of the COPAS Biosort for the distribution of individual worms with a liquid handling robot for sampling from populations, we were able to implement an efficient production workflow. To increase further the throughput of the procedure, a number of improvements could be envisioned. Currently, one of the limiting steps is the band-calling step. Since the *Mos1*-specific amplicon has a fixed size, it is likely that band detection could be completely automated. As it stands, on an average ~14 strains have to be screened to find one that is *Mos1*-positive. Clearly, any stable improvement in the overall yield would be helpful, but this has so far been elusive. Indeed, given the lack of strict correlation between observed transposition frequency and the proportion of *Mos1*-positive strains obtained at the end (Figure 7), it is not clear what parameters should be changed to boost the overall efficiency. A more radical amelioration would be the use of a transposable element containing a marker, such as a gene encoding a fluorescent protein, which would allow direct screening for individuals carrying transposon insertions. As the *Mos1* transposon can only carry short exogeneous DNA sequences (17), such an approach must await the development of other transposons able to carry larger inserts and compatible with germline transposition in *C.elegans*. Nevertheless, the results presented here clearly show that high-throughput methods can be developed using existing transposon technologies and successfully applied to a project that aims to benefit the increasing number of researchers who use *C.elegans* as a model.

The generation of a large collection of *Mos1* mutants is especially relevant now that techniques have been developed to exploit such a resource through engineering of the *C.elegans* genome by homologous recombination by another partner of the NEMAGENETAG consortium [V. Robert and J. L. Bessereau, personal communication (23), http://www.wormbase.org/db/misc/paper?name=WBPaper00027447]. The technique, termed MosTIC (*Mos1*-induced transgene-instructed gene conversion) allows (i) the introduction of point mutations, (ii) the engineering of deletions and (iii) the knock-in to a specific genomic locus of *gfp* at sites at least 500 bp on each side of a *Mos1* insertion site (Robert *et al*., manuscript submitted). Consequently, *Mos1* insertions in intronic and intergenic regions are potentially as useful as those that are within exons as the starting point for gene-specific recombineering.

Finally, this work clearly demonstrates that methods can be developed that allow the handling of very large numbers of nematode strains in parallel. Currently, we are treating up to 120 96-well plates per week (11 520 individual strains). These techniques could be readily adapted to numerous different experimental problems. For example, genetic screens for maternal or grand-maternal effect mutants are notoriously difficult because of their clonal nature. The procedure described here would be well suited to such screens. For reverse genetic genome-wide RNAi screens close to 20 000 clones need to be handled. Plate pouring and handling is often a limiting factor. Using the Biosort, such a screen could in theory be performed in <2 weeks.

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